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2. Patent application number *(The Patent Office will fill in this part)* — **9920074.3** — 24 AUG 1999

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THE BABRAHAM INSTITUTE
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Patents ADP number *(if you know it)* 7414519001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — ANIMAL MODEL AND USES THEREOF

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

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# ANIMAL MODEL AND USES THEREOF

The present invention relates to generation of animal models for pathologies involving monoamine dysfunction by means of manipulation of the vesicular monoamine transporter 2 (*VMAT2*) gene. Such models are useful particularly in study of various disorders, especially Parkinson's disease, schizophrenia and drug dependencies, and in assay methods for obtaining agents of therapeutic potential in such disorders.

More particularly, the present invention is based on work by the inventors who have generated transgenic mice which have profound changes in monoamine metabolism and yet unexpectedly survive into adulthood.

There are three major monoaminergic cell groups, ramifying extensively throughout the brain and distinguished by their neurotransmitter phenotype: noradrenaline (norepinephrine), dopamine and serotonin (Kandel et al. (1991) Principles of Neural Science (Appleton & Lange)). The vesicular monoamine transporter 2 (VMAT2) packages these monoamine neurotransmitters into vesicles after they have been synthesised from their amino acid precursors tyrosine and tryptophan, locally in the nerve terminal (Erickson et al. (1992) Proc. Natl. Acad. Sci. USA 89, 10993-10997). This sequestering action is important for normal neurotransmission and may also act to keep intracellular levels of the monoamine transmitters below toxic levels. The monoamines are

implicated in a variety of brain functions, encompassing sensory, motor, motivational and cognitive domains. There are indications that abnormalities in the functioning of these systems play key roles in the aetiology of a number of behavioural and cognitive disorders, including Parkinson's disease (PD) (Ehringer and Hornykiewics (1960) Wien Klin. Wochenschr. 38, 1236-1239), schizophrenia (Seeman et al. (1984) Science 225, 728-731) and drug addiction (Nestler and Aghajanian (1997) Science 278, 58-63).

Using genetic techniques the inventors have created a transgenic mouse with profound changes in monoamine metabolism that survives into adulthood. In the specific embodiments disclosed, VMAT2, the protein which sequesters monoamines intracellularly into vesicles, is no longer expressed in the animals. Neurochemically, the transgenic mice are characterised by low levels of tissue monoamines, including dopamine. These data are supplemented by behavioural findings providing indication of large disturbances in motor functioning. The mice are of immediate relevance to aspects of the motoric dysfunction found in Parkinson's disease, a condition in which dopamine neurons are selectively destroyed.

Animals provided in accordance with the present invention represent models for study of disorders such as Parkinson's disease and schizophrenia, and further provide for assays for identifying and obtaining agents which may be used for treatment of such disorders.

*Brief Description of the Figures*

Figure 1A shows a representation of the wild-type and predicted genomic sequences in the mouse genome after targeted insertion of the construct employed in making transgenic mice in accordance with an embodiment of the present invention. Prominent restriction endonuclease sites (B = BamHI, H = HindIII, K = KpnI, P = PflMI, X = XbaI, Xh = XhoI), neomycin resistance sequences (*neo*), thymidine kinase (*HSV tk*) and sequences recognised by the *Vmat2* 3' and 5' hybridisation probes (shown in Figure 1B) are indicated.

Figure 1B shows the *Vmat2* 3' and 5' hybridisation probes located in the third intron of the *Vmat2* gene, and shown to be unique in the mouse genome. the 5' probe is a 563 bp Saci/BamH1 fragment and the 3' probe is a 391 bp PVUII fragment consisting of a 200 bp PVUII and a 191 bp PVUII/SacII fragment radio-labelled by random priming.

Figures 2A and 2B show results of quantification of brain monoamines and metabolites in (from left to right in each group of bars) wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice. Noradrenaline, 5-hydroxyindolacetic acid (5-HIAA), dopamine, dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and homovanillic acid (HVA) levels were measured in and are shown for striatum (STR), cortex (CTX), substantia nigra (SN), hippocampus (HIP), cerebellum (CER) and

brainstem (BS). The results are represented as mean ± SEM.

Figure 3 shows results of quantification of monoamines and metabolites in the striatum of (from left to right in each group of bars) wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice.

Figures 4A, 4B and 4C show results of experiments in which ability of homozygous (-/-), heterozygous (+/-) and wild-type (+/+) mice to walk a 15mm round beam (Figure 4A), a 10mm round beam (Figure 4B) and a 10mm flat beam (Figure 4C) was assessed. The results show mean and SEM.

Figures 5A, 5B and 5C show results of experiments in which motivation, locomotion and rearing of homozygous (-/-), heterozygous (+/-) and wild-type (+/+) mice were assessed. Figure 5A shows number of visits into a novel place. Figure 5B shows number of rears in the novel place. Figure 5C shows time in the novel place. The results show mean and SEM.

Figure 6 shows amphetamine effects in (from left to right in each group of bars) wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice. Mean stereotypy scores are shown for 5 minute time points throughout an hour after intraperitoneal injection with a single dose of 3mg/kg d-amphetamine. Control mice were given saline.

The mouse *Vmat2* sequence has been published by Takahashi and

Uhl (1997) Mol. Brain Res. 49, 7-14. The rat *Vmat 2* sequence has been published by Liu et al. (1992) Cell 70, 539-551 and deposited in the EMBL/GenBank databases with accession number M97381, also published by Erickson et al. (1992) Proc. Natl. Acad. Sci. USA 89, 10993-10997 and deposited under accession number L00603. The rat sequence is also disclosed in US-A-5688936. Homologous genes have been cloned in human (Xu et al. (1997) Mol. Brain Res. 45, 41-49, accession numbers X94079 and X94080), bovine (Krejci et al. (1993) FEBS Lett. 335, 27-32, accession number X76380), in the marine ray Torpedo (Varoqui et al. (1994) FEBS Lett. 342, 97-102, accession number S43686) and in *Caenorhabditis elegans* (Wilson et al. (1994) Nature 368, 32-38, accession number AAA82627).

The invention provides for manipulation of nucleic acid in order to modify cells of an animal. Heterologous nucleic acid may be integrated into the genome (e.g. chromosome) of cells. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

For information on manipulation and use of nucleic acid see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are

described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1994. Standard references for generation of transgenic animals include Hogan et al., *Manipulating the Mouse Embryo* (Cold Spring Harbor, Laboratory, Cold Spring Harbor, NY, 1986), Grosveld and Kollias, 1992, *Transgenic Animals* (Academic Press Limited, London), and Joyner, 1995, *Gene Targeting A Practical Approach* (Oxford University Press).

A modified animal cell, for instance with a *VMAT2* knock-out or mutation, may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse. Genetically modified or transgenic animals are provided as further aspects of the present invention. Preferred animals of the invention are rodent, such as mouse or rat, preferably mouse.

In various further aspects, the present invention provides a non-human animal with a modified *VMAT2* transgene within its genome.

The presence of a mutant, allele or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, allow for the organism to be used as a model in testing and/or studying the role of the

gene or substances which modulate activity normally provided by VMAT2 or are otherwise indicated to be of therapeutic potential. This is discussed further below.

Important aspects of the present invention are based on the inventors' construction of animals with *VMAT2* deficiency, useful as models for disorders in which monoamines are depleted, especially Parkinson's disease, schizophrenia and drug addictions. The experimental work included below indicates that not only can *VMAT2* knockout mice (unexpectedly) survive to adulthood but also that they exhibit physiological and behavioural abnormalities that mimic well those seen in sufferers of Parkinson's disease. Furthermore, the monoamines, and in particular dopamine, are implicated in the expression of the cognitive and behavioural deficits observed in schizophrenia. Assessment of the mice in behavioural paradigms testing attentional functioning (Pre-pulse inhibition, Latent inhibition) and perseverative responding (reversal learning) may be used in modelling of schizophrenia. This may include tests of attentional functioning such as pre-pulse inhibition (which assesses the ability of a subject to filter salient sensory, usually auditory, stimuli) and latent inhibition (which is held to test ability of a subject to ignore irrelevant information about the environment). The behavioural rigidity (perseverative) aspects of schizophrenia may be tested using simple reversal learning paradigms where the subject must inhibit previous learning in order to take on board new learning (such as in the case where originally A was

correct and B incorrect, but now B is correct and A is incorrect). The monoamines are also heavily implicated in the acute reinforcing properties of drugs of abuse such as cocaine, so mice may be assessed in paradigms such as the conditioned place preference which index the reinforcing properties of drugs of abuse.

Animal models for *VMAT2* deficiency may be constructed using standard techniques for introducing mutations into an animal germ-line. In one example of this approach, using a mouse, a vector carrying an insertional mutation within the gene may be transfected into embryonic stem cells. A selectable marker, for example an antibiotic resistance gene such as *neoR*, may be included to facilitate selection of clones in which the mutant gene has replaced the endogenous wild type homologue. Such clones may be also be identified or further investigated by Southern blot hybridisation. The clones may then be expanded and cells injected into mouse blastocyst stage embryos. Mice in which the injected cells have contributed to the development of the mouse may be identified by Southern blotting. These chimeric mice may then be bred to produce mice which carry one copy of the mutation in the germ line. These heterozygous mutant animals may then be bred to produce mice carrying mutations in the gene homozygously. The mice having a heterozygous mutation in the gene may be a suitable model for human individuals having one copy of the gene mutated in the germ line who are at risk of developing a disorder or disease.

The invention therefore further provides a non-human transgenic animal which harbours at least one copy of a *VMAT2* transgene, especially a *VMAT2* sequence disrupted to prevent or inhibit production of functional VMAT2 protein, integrated into a chromosomal location. Animals according to the invention are preferably homozygous for *VMAT2* mutation, preferably homozygous for a *VMAT2* null allele.

In a further aspect the invention provides a non-human transgenic animal which harbours one or more integrated constructs or targeted mutations that disrupt the function of endogenous *VMAT2* gene. Such animals are referred to as "knock-outs", although it is not required by this aspect of the invention that function be totally ablated (this may be preferred). The invention provides a non-human animal with at least one inactivated endogenous *VMAT2* allele, and which is preferably homozygous for inactivated *VMAT2* alleles.

Various approaches for targeting constructs or mutations (generally deletions) to chromosomal locations are available in the art, and generally make use of homologous recombination between a target sequence in the chromosome and a region in a vector which is substantially complementary to the target sequence. Sequences flanking a target gene or a portion thereof may be employed, allowing for deletion of the target gene or the relevant portion.

Transgenic mutations, including deletions, in a gene locus in accordance with the present invention may be detected using conventional techniques, such as fluorescent in situ hybridisation (FISH) with appropriate probes appropriately labelled, PCR analysis etc.

Further aspects of the present invention provide cells of transgenic animals as disclosed, whether isolated cells or cell lines derived from the animals and optionally immortalised using standard techniques.

A further aspect of the present invention provides a method of making a transgenic animal with a *VMAT2* mutation that disrupts VMAT2 function, either preventing or inhibiting production of VMAT2 protein, or abolishing or inhibiting VMAT2 protein function, i.e. ability to package noradrenaline (norepinephrine), dopamine and serotonin monoamine neurotransmitters into vesicles, the method comprising: providing such a mutation in a *VMAT2* sequence in an embryonic stem cell, injecting the embryonic stem cell into a host blastocyst, developing the blastocyst through embryogenesis and generating an animal. Blastocysts may be developed in the uteri of pseudopregnant nonhuman females and chimeric animals may be born. Animals homozygous for *VMAT2* mutation may be generated by backcrossing. Preferably an animal (e.g. mouse) generated in accordance with the present invention matures to adulthood. A mutation within a *VMAT2* sequence that disrupts

its function may be a deletion, and may be introduced by a process comprising homologus recombination.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in treating or preventing a disorder or disease is provided by animals according to the present invention. Substances identified as able to influence physiological or behavioural function of *VMAT2* mutant animals represent an advance in the fight against disease since they provide basis for design and investigation of therapeutics for *in vivo* use. Furthermore, they may be useful in any of a number of conditions, including diseases and disorders involving monoamine dysfunction, such as Parkinson's disease, schizophrenia and drug addictions.

In various further aspects the present invention relates to screening and assay methods and means, and substances identified thereby.

An animal model according to the present invention may be employed in assay methods for molecules of therapeutic

potential.

A further aspect of the present invention provides an assay method which comprises:

(a) treating an animal model as disclosed herein with a test substance;

(b) determining the presence or absence of an effect on the animal as a result of the treatment with the test substance.

The nature of an effect when detected may be investigated. Potential end-points for detection include visual effects, effects determined immunologically or biochemically, and effects determined by means of determination of gene expression, for instance by means of Southern or Northern blotting of nucleic acid extracts or derivatives from appropriate cells. The skilled person is well aware of the need for control experiments and well able to design appropriate controls, both positive and negative. An end-point indicative of a positive result may be chosen in view of the therapeutic application in mind.

Behavioural end-points indicative of potential therapeutic application of an agent include: amelioration of "spontaneous" (i.e. non-drug induced) deficits in motor and cognitive abilities as indexed by locomotor scores, beam walking, syntactic grooming, pre-pulse inhibition, reversal learning and more sophisticated tests of motor readiness such as those indexed by simple and choice reaction-time tasks. A second

category of behavioural end-points involve amelioration of drug-induced changes; including d-amphetamine effects on turning and stereotyped behaviours. These end-points are most relevant to modelling of symptoms of Parkinson's disease and schizophrenia. Behavioural end-points more directly relevant to addiction include drug conditioned place preference, self-administration paradigms and progressive ratio tasks.

Test agents may reduce the animal's supersensitivity to drugs of addiction such as cocaine and amphetamine, and agents may be assessed for ability to reduce the stereotypy produced by such addictive drugs.

The animal model may be used to test effects of transplantation of cell lines or vehicles such as polymer implants designed to release dopamine for treatment of Parkinsonism, or to test effects of somatic gene therapy, such as proposed with Herpes- or adeno-associated virus vectors (During et al. (1994) Science 266, 1399-1402; Szczypka et al. (1999) Neuron 22, 167-178).

VMAT2 is involved in safe storage and inactivation of neurotoxins such as MPTP, so the mice may be used to provide a screen for behavioural and toxic effects of compounds which require *VMAT2* for inactivation. Toxicity may be assessed by acute loss of dopamine cells (e.g. by cell count after histology, worsening of motor deficits of the treated

animals).

Similarly, cultured monoaminergic cells (noradrenaline, serotonin or dopamine cells) from these animals may be used to test toxicity of toxins normally inactivated by VMAT2.

The purpose of an assay employing an animal model in accordance with the invention may be for identifying or obtaining an agent with therapeutic potential in treatment of a disorder resulting from a defect in *VMAT2* function. Such disorders may include any mentioned herein.

Test compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes, combinatorial libraries and so on. Extracts of plants which contain several characterised or uncharacterised components may also be used. A further class of putative inhibitor compounds can be derived from the polypeptide encoded by the gene manipulated in the model (VMAT2). Peptide fragments of from 5 to 40 amino acids, for example from 6 to 10 amino acids from the region of the relevant polypeptide responsible for interaction with another molecule, may be tested for their ability to disrupt such interaction. Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge

characteristics.

An animal may be treated with a test substance at an appropriate dosage, depending on the site of administration, any known potency of the substance, solubility and other factors routinely taken into account by those skilled in the art.

Similar assay methods may employ cells or cell lines derived from transgenic animals (including knock-outs) generated as described further elsewhere herein.

Following identification of a substance which provides a positive effect, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

Thus, the present invention extends in various aspects not only to a substance identified as of interest or therapeutic potential in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a disorder or disease, use of such a substance in manufacture of a composition for administration, e.g. for treatment of a

disorder or disease, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Disorders and diseases which may be treated in accordance with aspects of the present invention have been discussed and mentioned already herein.

A substance identified using as a modulator of polypeptide or promoter function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use. The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides may not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property.

Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and

does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of substances identified as having ability to modulate polypeptide or activity using a screening method as disclosed herein are included within the scope of the present invention. A polypeptide, peptide or substance able to modulate activity of a polypeptide according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

Administration to an individual of a therapeutically useful molecule identified in accordance with the present invention is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous

solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Aspects of the present invention will now be illustrated with reference to the accompanying figures described already above and experimental exemplification, by way of example and not limitation. Further aspects and embodiments will be apparent to those of ordinary skill in the art. All documents mentioned in this specification are hereby incorporated herein by reference.

*EXAMPLE 1 - Generation of Vmat2 Knockout Mice*

*VMAT2* knockout mice were generated using a replacement vector containing sequences homologous to the *VMAT2* target sequences with a neo expression cassette inserted within the region of homology (Figure 1).

*VMAT2* transgenic mice were produced as follows. Several overlapping *VMAT2* genomic Lambda clones were isolated from a 129 mouse library in λ 2001 with a murine *VMAT2* cDNA probe radiolabelled and hybridised to $10^7$ clones. To obtain this

probe, single stranded cDNA was synthesised from 5μg total mouse brain RNA by PCR using the oligonucleotides 5'-CGTGTTCCTTGCGCTGCTGCTGGA-3' and 5'-CTGAAGGGACCTGGCGATCAGCAG-3', corresponding to previously reported rat *VMAT2* cDNA sequences (Liu et al. (1992) Cell 70, 539-551; Erickson et al. (1992) Proc. Natl. Acad. Sci. USA 89, 10993-10997). Genomic SacI and HIII subclones were generated in pBluescript (Stratagene). The mutation was generated with a targeting vector containing sequences homologous to the *VMAT2* target sequences with a neo expression cassette inserted within the region of homology (Figure 1A).

The *VMAT2* targeting vector was constructed by cloning the β-actin neo cassette into the BHI site of a 2.5 kb HIII-BHI fragment containing the *VMAT2* promoter and the leader exon cloned into pBluescript (Stratagene). A 2.2kb PVUII fragment from the third intron of the *VMAT2* gene was cloned into the blunt ended NotI site of this construct. The HSV-tk gene (Mansour et al. (1988) Nature 336: 348-352) used for negative selection was cloned into the Bg1II site on the 3' end of the construct. Thirty micrograms of the targeting construct were linearised with Sa1I and transfected by electroporation into CGR embryonic stem cells. CGR cells were selected for homologous recombination by culture in DMEM containing 15% fetal calf serum (Sigma), 0.1mM 2-mercaptoethanol, 500μg/ml G418, and 2μm gancyclovir on days 2-7 after transfection. G418- and gancyclovir-resistant colonies were picked on day 8.

HIII/XbaI digests of DNA prepared from 325 resistant clones were screened by Southern blot analysis using a 200-bp PVUII/SacII fragment flanking the site of recombination and radiolabelled by random priming as a hybridisation probe. Six positive cell lines from the double resistant colonies displayed the 6.4-kb HIII fragment, missing the XbaI site, anticipated of wild-type clearly distinguishable from the 3.5 kb HIII/XbaI fragment obtained from homologous recombinants DNA (Figures 1A and B).

Chimeric mice were generated by injecting 15-20 stem cells of one of the positive clones into blastocysts harvested from *C57BL/6* mice and implanting the blastocysts into uteri of pseudopregnant *C57 BL/6* females 2.5 days postcoitus. Southern blot analysis of DNA extracted from tail-tip specimens of agouti offspring of the chimeras revealed that germ-line transmission was achieved from crosses between two chimeric animals, GB1/1 (female) and KA1 (male) with a BL/6 male and female. The GB1/1 homologous recombination event represented a double reciprocal recombination resulting in a deletion of exons 1 and 2 and flanking intron sequences and a replacement of the deleted region with the neo cassette. The homologous recombination event in KA1, the line of interest, represented a single reciprocal recombination that occurred only on one arm of the construct as shown in Figure 1A resulting in an insertion of the neo cassette and the leader exon with 5' flanking sequences into the third intron of the *VMAT2* gene

instead of a recombination of the homologous 5' regions of targeting vector and mouse genomic sequences, that would have resulted into the deletion that was observed in the GB1/1 line.

The gene replacement technique is one of the more recent strategies to introduce defined mutations into a gene of interest. A number of clones targeted with replacement vectors do not recombine as predicted, but often integration events occur (Schwartzberg et al. (1990) Proc. Natl. Acad. Sci. USA 87, 3210-3214; Thomas and Capecchi (1986) Nature (London) 324, 34-38). A report on the target frequency and integration pattern of replacement vectors for the HPRT gene shows that most of the clones targeted with replacement vectors were not generated by the predicted gene replacement event; rather the entire construct was usually integrated into the *hprt* locus (Hasty et al. (1991) Mol. Cell. Biol. 11:5586-5591). By generating the KA1 mutation, the ability of the targeting construct to recombine only with one of its arms was used to insert the promoter region including the leader exon of the *VMAT2* gene into its third intron (Figure 1A).

Unique probes from 5' and 3' flanking sequences of the transgene insertion site (Figure 1B) show the polymorphism caused by the *VMAT2* mutation in the Southern blot analysis using transgenic (-/- and +/-) and wild type (+/+) tail DNA digested with different restriction enzymes that do cut

(BamHI, XhoI, XbaI and PflmI) or do not cut (HindIII and KpnI) in the transgene. The insertion event deleted 245 bp from the transgene 5' end, whereas the homologous recombination that occurred with the sequences 3' of the neo gene left that part of the transgene intact. To verify this deletion, primers were designed to PCR the junction between transgene and 5' flanking sequence. The sequence comparison of the PCR products with the cloned genomic region revealed the location of the insertion as well as the extent of the 5' deletion.

A double reciprocal recombination results in a deletion of exons 1 and 2 and flanking intron sequences and a replacement of the deleted region with the *neo* cassette. A number of clones targeted with replacement vectors do not recombine as predicted, but often integration events occur (Thomas and Capecchi (1986) Nature (London) 324, 34-38; Schwartzberg et al. (1990) Proc. Natl. Acad. Sci. USA 87, 3210-3214). A single reciprocal recombination that occurs only on one arm of the construct as shown in Figure 1 results in an insertion of the neo cassette and the leader exon with 5' flanking sequences into the third intron of the *VMAT2* gene. Mice that were derived from these clones were able to survive with the mutation on both alleles of the *VMAT2* gene.

Unique probes from 5' and 3' flanking sequences of the transgene insertion site (5' probe and 3' probe sequences are shown in Figure 1B) showed the polymorphism caused by the

*VMAT2* mutation in Southern blot analysis using transgenic (-/- and +/-) and wild type (+/+) tail DNA digested with different restriction enzymes that do cut (BamHI, XhoI, XbaI and PflmI) or do not cut (HindIII and KpnI) in the transgene. The insertion event deleted 245 bp from the transgene 5' end, whereas the homologous recombination that occurred with the sequences 3' of the *neo* gene left that part of the transgene intact. To verify this deletion, primers SF1 and SR1 and HR2 were designed to PCR the junction between transgene and 5' flanking sequence, having the following sequences:

SF1: 5'-GGCTGATTGTGCACCCACAGAGAAGCCC-3'

SR1: 5'-GCCACTTCACAATGGGGGAGGAGGC-3'

HR2: 5'-CGCCAAAGGAACCCCAAGTAAATCC-3'

PCR products SF1-HR2 and SF1-SR1 from amplification of homozygous (-/-), heterozygous (+/-) and wild-type (+/+) tail DNA with these primers were displayed on an ethidium bromide stained gel. No amplification products were produced in wild-type DNA. The insertion of the targeting vector generated additional binding sites for the primers HR2 and SR1 downstream of SF1, allowing amplification of the transgenic alleles with primer pairs SF1-HR2 and SF1-SR1 only in heterozygous and wild-type DNA.

The sequence comparison of the PCR products with the cloned genomic region revealed the location of the insertion as well as the extent of the 5' deletion. The sequence of the longer

PCR product illustrated the junction between transgene and genome in the *VMAT2* mutants down to the nucleotide level.

*VMAT2* expression from the mutated allele was analysed by comparing *Vmat2* mRNA levels in total RNA preparations from the substantia nigra of homozygous and wild type mice. No *Vmat2* mRNA was detected by Northern blot analysis in the mutant mice. Total RNA from the substantia nigra, the striatum and the cortex of 6 month old wild-type (+/+) and homozygous (-/-) mice was prepared, blotted on to a membrane and hybridised to a 626 bp *Vmat2* specific cDNA probe including sequences from the leader exon and exons 1 to 4. Only the substantia nigra of wild-type mice was found to express detectable amounts of *Vmat2* message.

*In situ* hybridisation of brain sections with oligonucleotides complementary to the *Vmat2* message confirmed the absence of the *Vmat2* mRNA expression in the substantia nigra of homozygous brains. Additionally, no *Vmat2* message was found in the raphe nuclei of homozygous animals by this technique.

Western blot analysis of striatal membranes from mutant and wild type mice was not able to pick up VMAT2 protein among striatal membrane proteins from homozygous mice. ECL Western blot analysis was performed with a polyclonal antiserum raised against a synthetic peptide from the intracellular C-terminal

region of the human VMAT2 protein, dilute 1:1000. These antibodies detected two background bands of higher molecular weight (≥80 kDa) in both samples. The smaller VMAT2 band was only detectable in the wild-type mouse and absent in the homozygous *VMAT2* mutant.

To further assess the extent of the loss of Vmat2 message a more sensitive approach, an RT PCR was performed for detecting messenger RNA from the mutated locus. Because the promoter region inserted into the third intron of the Vmat2 gene a shorter message could be initiated from this now further downstream located Vmat2 promoter containing the leader exon of the gene. To test this, a leader exon primer and primers complementary to exon 4 and exon 12 sequences (Takahashi and Uhl (1997) Mol. Brain Res. 49, 7-14) were designed with the following sequences:

leader exon primer: 5'-CCCTGCAGGCAGTCGCAGGCGAGC-3'
exon 4 primer: 5'-CTGCAGGGACCTGGCGATCAGCAGG-3'
exon 12 primer: 5'-CCAACTCCAAAGTTGGGAGCGATGAG-3'

These primers amplify a message in total brain cDNA from homozygous mice (-/-) which is 485 bp shorter than the original wild type (+/+) products and sequence analysis reveals that these shorter products are depleted of the sequences coding for the first and second exon. This shorter message does not appear to be stable enough to detect it by Northern blot analyses and *in situ* hybridisation techniques

and it seems extremely unlikely that this message contributes to any translation products, as these are not detectable in the Western blots.

*EXAMPLE 2 - Phenotypic Analysis of Vmat2 Knockout Mice Indicating Utility As A Model of Parkinson's Disease and Other Disorders*

The inventors assessed the brain and behavioural phenotypes of the *VMAT2* knockout mice.

These mice showed profound reductions in brain levels of the monoamines, especially dopamine (Figure 2A, 2B).

Dopamine, noradrenaline and serotonin as well as their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA) were measured in perchloric acid extracts prepared from brain areas of *VMAT2* +/+ (n=13, SN n=11; Cer n=12; BS n=8), *VMAT2* +/- (n=17), and *VMAT2* -/- (STR, CER, CTX n=6; SN, HIP n=5, BS n=3) mice using HPLC with electrochemical detection. Relative to the brains of wild-type animals, the brains of *VMAT2* homozygous mice show drastic reductions in the levels of all three monoamines. For example, dopamine levels are significantly up to 86.5% reduced in the striatum ($p<0.001$), in the cortex ($p<0.002$) and in the substantia nigra ($p<0.04$). Noradrenaline levels are up to 77% significantly reduced in

the striatum ($p<0.04$), the substantia nigra ($p<0.001$), the hippocampus ($p<0.014$), the cortex ($p<0.020$) and the cerebellum ($p<0.001$). Serotonin levels are significantly reduced by up to 78% in the striatum ($p<0.017$) and in the cortex ($p<0.001$) as well as in the cerebellum ($p<0.005$) and the hippocampus ($p<0.023$). In contrast metabolite levels appear similar in all three groups. The brains of heterozygous animals generally do not show significant reductions in monoamine levels compared to their wild-type littermates.

Similar progressive changes accompany the nigral degeneration in Parkinson's disease: the concentration of the catecholamines dopamine and noradrenaline in the striatum significantly decrease and as a consequence also the concentrations of the acidic metabolites of dopamine HVA and DOPAC decline. This monoamine deficiency then causes motor disturbances observed in the advanced stages of the disease. The mouse mutants show the loss of striatal monoamines and its metabolites (Figure 3) without the anterograde degeneration of the nigrostriatal fibers, suggesting that low monoamine levels do not precede nigrostriatal degeneration. This significant drop in monoamines certainly contributes to the co-ordinated movement deficits in the mice as indexed by beam walking (Figures 4A, 4B and 4C and Tables 1, 2 and 3).

The animals were taught to walk across beams of 15mm and 10mm round, and 10mm flat in sessions 1 to 3. These sessions lasted for three days, letting them do three crossings per

beam type. Beam walking sessions 4 and 5 done on two successive days included the round experimental beam with 5mm in diameter. The results for each beam shown in Figures 4A, 4B and 4C and Tables 1, 2 and 3 are the mean values ±SEM of six crossings done by 15 mice of each genotype in sessions 4 and 5. The data were analysed by Tukey-Kramer Multiple Comparisons Test: for (15mm round) $P<0.01$ comparing homozygous with wild-type littermates; for (10mm round) $P<0.01$ comparing homozygous with wild-type littermates; for (10mm flat) $P<0.05$ comparing homozygous with heterozygous littermates and $P<0.01$ comparing homozygous with wild-type littermates.

The most incapacitating symptoms in Parkinson's disease are the slowing down in movement (bradykinesia) leading to akinesia and rigidity (Stern and Lees (1990) Parkinson's Disease, The Facts (Oxford University Press)). The mice show a substantial loss in activity as measured by the number of visits and rears in the novelty place preference test illustrated in Figures 5A, 5B and 5C and Tables 4, 5 and 6, mimicking the Parkinson's disease symptom of bradykinesia, but the mutation does not effect motivation, as the mutants spend a similar amount of time in the novel chamber as their wild type litter mates (Figures 5A, 5B and 5C and Tables 4, 5 and 6).

Homozygous, heterozygous and wild-type mice were given the option to go to a novel chamber after 60 minutes habituation in a familiar chamber. Given are the number of visits into

the novel place, the time spent in the novel place and the number of rears they had in the novel place. The mean values ±SEM for 15 mice of each genotype are shown. The data were analysed by Tukey-Kramer Multiple Comparisons Test: for the number of visits $P<0.001$, comparing homozygous with wild-type littermates and homozygous with heterozygous littermates; for the time spent in the novel place the differences between the 3 groups are not significant; for the number of rears $P<0.05$ comparing homozygous with heterozygous littermates and $P<0.01$ comparing homozygous with wild-type littermates.

The results show that the *VMAT2* mutation does not affect motivation, but interferes with locomotion and rearing.

Catecholamines, which include dopamine noradrenaline and serotonin are not only important neurotransmitters, that regulate motor coordination, but also are essential for mouse fetal development and postnatal survival (Zhou et al. (1995) Nature (London) 374, 640-643; Zhou and Palmiter (1995) Cell 83, 1197-1209; Ungerstedt (1971) Acta Physiol. Scand. (Suppl.) 367, 49-122). The mutant mice show a higher postnatal lethality rate (about 1 in eight homozygous offspring die after birth) and also reduced weight gain.

Amphetamine effects in homozygous (-/-), heterozygous (+/-) and wild-type (+/+) mice were analysed by stereotypy ratings scored for 1 hour after mice were injected intraperitoneally with a single dose of either 3mg/kg d-amphetamine or saline.

The greater was the score the greater was the extent of stereotyped behaviour observed. Eleven wild-type, 8 heterozygous and 9 homozygous mice were included in the experiment. The data indicate a dramatically increased response to d-amphetamine, an indirect dopamine agonist, in the homozygous mice (Figure 6). This indicates that impaired synaptic vesicle function produces compensatory, monoamine receptor sensitising modifications on the postsynaptic site of the basal ganglia. This is a well known response, called drug supersensitivity, that is also observed in the course of acute L-DOPA treatment of Parkinson's disease. The common use of L-DOPA for the treatment of Parkinson's disease showed that it stimulates directly striatal D1 and D2 receptors which, by virtue of the death of nigrostriatal neurons, show denervation supersensitivity, meaning that severely effected patients respond more to L-DOPA than less severely effected patients, presumably as they have a greater degree of denervation supersensitivity (Hopkins (1991) Clinical Neurology, A Modern Approach (Oxford University Press)).

Hence, the data clearly provide indication of the utility of the mice as a model of Parkinson's disease particularly as regarding the deficits in the initiation and execution of willed movements, shown to be symptomatic for this disorder.

These mice may also provide new information about the contribution of vesicular function in the action of monoaminergic drugs, such as neuroleptics, and neurotoxins.

Neuroleptics or antipsychotic drugs (Julien (1995) A primer of drug action: a concise, nontechnical guide to the actions, uses, and side effect of psychoactive drugs. 7th edition, W.H. Freeman & Company) are used for the treatment of schizophrenia, a very common disorder with 1/100 experiencing episodes of schizophrenia once in their life time. These drugs often cause side effects resembling Parkinsonian symptoms after long term treatment. Standard neuroleptics are potent inhibitors of D2 like receptors. The inhibition of D2 and D4 receptors by neuroleptics in the caudate nucleus and the putamen or corpus striatum especially cause motoric disturbances mimicking the Parkinsonian symptoms of tremor, rigidity and bradykinesia. The extent of the side effects depends on the dose of the neuroleptic and side effects usually disappear after the medication is stopped. The mice offer a model system to test for drugs to treat these disabling side effects in patients. Neurotoxins, such as MPTP produce Parkinsonian symptoms in man as well as in mice (Davis et al. (1979) Psychiatry Res. 1, 249-254; Langston et al. (1983) Science 219, 979-980; Duvoisin et al. (1984) Dopaminergic Neurotoxicity of MPTP in the Mouse. Recent Developments in Parkinson's Disease, edited by S. Fahn, et al. (Raven Press), 147-154.). The mutants now offer a system to examine the contribution of vesicular action to the toxic effects of MPTP and other neurotoxins.

**Table 1**

| Title | beam walking 15mm round | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 73.3 | 4 | 3.6 |
| 2 | 69 | 4 | 3.6 |
| 3 | 6 | 10.3 | 2 |
| 4 | 2.6 | 31.3 | 6.3 |
| 5 | 6.3 | 6.3 | 5 |
| 6 | 12 | 17.3 | 4.6 |
| 7 | 55 | 2.6 | 11.3 |
| 8 | 10.3 | 37 | 6.6 |
| 9 | 8.6 | 16 | 10.3 |
| 10 | 12.3 | 20 | 9 |
| 11 | 22 | 2.6 | 3.3 |
| 12 | 5 | 19 | 3.6 |
| 13 | 3 | 15 | 4.6 |
| 14 | 20.3 | 7.3 | 4 |
| 15 | 43 | 1.6 | 2.6 |
| Mean | 23.25 | 12.95 | 5.36 |
| SEM | 6.32 | 2.78 | 0.72682 |
| Sample size | 15 | 15 | 15 |
| SD | 24.48 | 10.76 | 2.81 |
| 95% CI min | 9.69 | 7.00 | 3.80 |
| 95% CI max | 36.80 | 18.91 | 6.92 |
| Minimum | 2.60 | 1.60 | 2.00 |
| Maximum | 73.30 | 37.00 | 11.30 |

**Table 2**

| Title | beam walking 10mm flat | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 90 | 6 | 7.3 |
| 2 | 90 | 3.3 | 2.6 |
| 3 | 12 | 18 | 2.3 |
| 4 | 5.3 | 53 | 4 |
| 5 | 11 | 5 | 2 |
| 6 | 26.6 | 10.3 | 6 |
| 7 | 79 | 3.3 | 4.3 |
| 8 | 9.6 | 30.3 | 4.6 |
| 9 | 34.3 | 4.6 | 21.3 |
| 10 | 12.6 | 19 | 16 |
| 11 | 23.3 | 3 | 5.3 |
| 12 | 9.6 | 8 | 3.6 |
| 13 | 5.6 | 10 | 8.3 |
| 14 | 32 | 17 | 3.6 |
| 15 | 67.3 | 2.6 | 2.3 |
| Mean | 33.88 | 12.89 | 6.23 |
| SEM | 8.13 | 3.52 | 1.41 |
| Sample size | 15 | 15 | 15 |
| SD | 31.48 | 13.62 | 5.45 |
| 95% CI min | 16.45 | 5.35 | 3.21 |
| 95% CI max | 51.31 | 20.44 | 9.25 |
| Minimum | 5.30 | 2.60 | 2.00 |
| Maximum | 90.00 | 53.00 | 21.30 |

**Table 3**

| Title | beam walking 10mm round | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 52.5 | 5.3 | 8.6 |
| 2 | 90 | 6.3 | 4 |
| 3 | 15.3 | 14 | 3 |
| 4 | 4.6 | 41.3 | 5.3 |
| 5 | 7 | 7.6 | 4.3 |
| 6 | 64 | 9 | 4.6 |
| 7 | 90 | 3.3 | 14 |
| 8 | 12 | 73.3 | 10.3 |
| 9 | 16 | 18.3 | 24 |
| 10 | 9.3 | 27.6 | 28.6 |
| 11 | 15.3 | 3.3 | 5 |
| 12 | 9.6 | 18 | 3 |
| 13 | 8.3 | 5.6 | 9.6 |
| 14 | 34 | 13.3 | 5.3 |
| 15 | 90 | 5 | 2.3 |
| Mean | 34.53 | 16.75 | 8.79 |
| SEM | 8.62 | 4.86 | 2.03 |
| Sample size | 15 | 15 | 15 |
| SD | 33.39 | 18.80 | 7.85 |
| 95% CI min | 16.03 | 6.33 | 4.45 |
| 95% CI max | 53.02 | 27.16 | 13.14 |
| Minimum | 4.60 | 3.30 | 2.30 |
| Maximum | 90.00 | 73.30 | 28.60 |

**Table 4**

| Title | visits into novel place | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 11 | 15 | 17 |
| 2 | 16 | 9 | 19 |
| 3 | 7 | 18 | 11 |
| 4 | 10 | 24 | 11 |
| 5 | 8 | 12 | 17 |
| 6 | 9 | 15 | 11 |
| 7 | 11 | 18 | 23 |
| 8 | 13 | 16 | 19 |
| 9 | 6 | 15 | 13 |
| 10 | 12 | 18 | 11 |
| 11 | 7 | 8 | 7 |
| 12 | 7 | 19 | 19 |
| 13 | 9 | 17 | 25 |
| 14 | 5 | 18 | 16 |
| 15 | 11 | | 14 |
| 16 | 11 | | |
| Mean | 9.56 | 15.86 | 15.53 |
| SEM | 0.718614 | 1.10 | 1.29 |
| Sample size | 16 | 14 | 15 |
| SD | 2.87 | 4.13 | 5.00 |
| 95% CI min | 8.03 | 13.47 | 12.77 |
| 95% CI max | 11.09 | 18.24 | 18.30 |
| Minimum | 5.00 | 8.00 | 7.00 |
| Maximum | 16.00 | 24.00 | 25.00 |

Table 5

| Title | rears in novel place | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 2 | 46 | 66 |
| 2 | 56 | 42 | 31 |
| 3 | 37 | 33 | 56 |
| 4 | 27 | 61 | 21 |
| 5 | 38 | 77 | 9 |
| 6 | 20 | 46 | 32 |
| 7 | 50 | 47 | 58 |
| 8 | 27 | 22 | 30 |
| 9 | 20 | 33 | 59 |
| 10 | 53 | 45 | 70 |
| 11 | 18 | 20 | 54 |
| 12 | 13 | 45 | 34 |
| 13 | 27 | 37 | 58 |
| 14 | 2 | 37 | 79 |
| 15 | 18 | | 37 |
| 16 | 11 | | |
| Mean | 26.19 | 42.21 | 46.27 |
| SEM | 4.19 | 3.89 | 5.16 |
| Sample size | 16 | 14 | 15 |
| SD | 16.77 | 14.56 | 19.97 |
| 95% CI min | 17.25 | 33.81 | 35.21 |
| 95% CI max | 35.12 | 50.62 | 57.32 |
| Minimum | 2.00 | 20.00 | 9.00 |
| Maximum | 56.00 | 77.00 | 79.00 |

Table 6

| Title | time in novel place | | |
|---|---|---|---|
| Column ID | A | B | C |
| Column Title | homo | hetero | wild |
| Raw or Mean | Raw Data | Raw Data | Raw Data |
| 1 | 6.24 | 6.31 | 5.01 |
| 2 | 7.31 | 8.04 | 6.41 |
| 3 | 4.17 | 8.02 | 7.19 |
| 4 | 9.12 | 7.20 | 6.13 |
| 5 | 5.50 | 7.10 | 7.54 |
| 6 | 5.48 | 6.31 | 6.29 |
| 7 | 2.54 | 8 | 8.48 |
| 8 | 6.23 | 8.47 | 5.19 |
| 9 | 8.19 | 6.13 | 5.22 |
| 10 | 9.14 | 8.27 | 5.14 |
| 11 | 8.38 | 8.44 | 7.58 |
| 12 | 8.15 | 8.12 | 5.23 |
| 13 | 8.18 | 8.32 | 8.07 |
| 14 | 8.05 | 5.25 | 7.46 |
| 15 | 8.5 | | 8.33 |
| 16 | 8.23 | | |
| Mean | 7.09 | 7.43 | 6.62 |
| SEM | 0.472485 | 0.279653 | 0.326579 |
| Sample size | 16 | 14 | 15 |
| SD | 1.89 | 1.05 | 1.26 |
| 95% CI min | 6.08 | 6.82 | 5.92 |
| 95% CI max | 8.09 | 8.03 | 7.32 |
| Minimum | 2.54 | 5.25 | 5.01 |
| Maximum | 9.14 | 8.47 | 8.48 |

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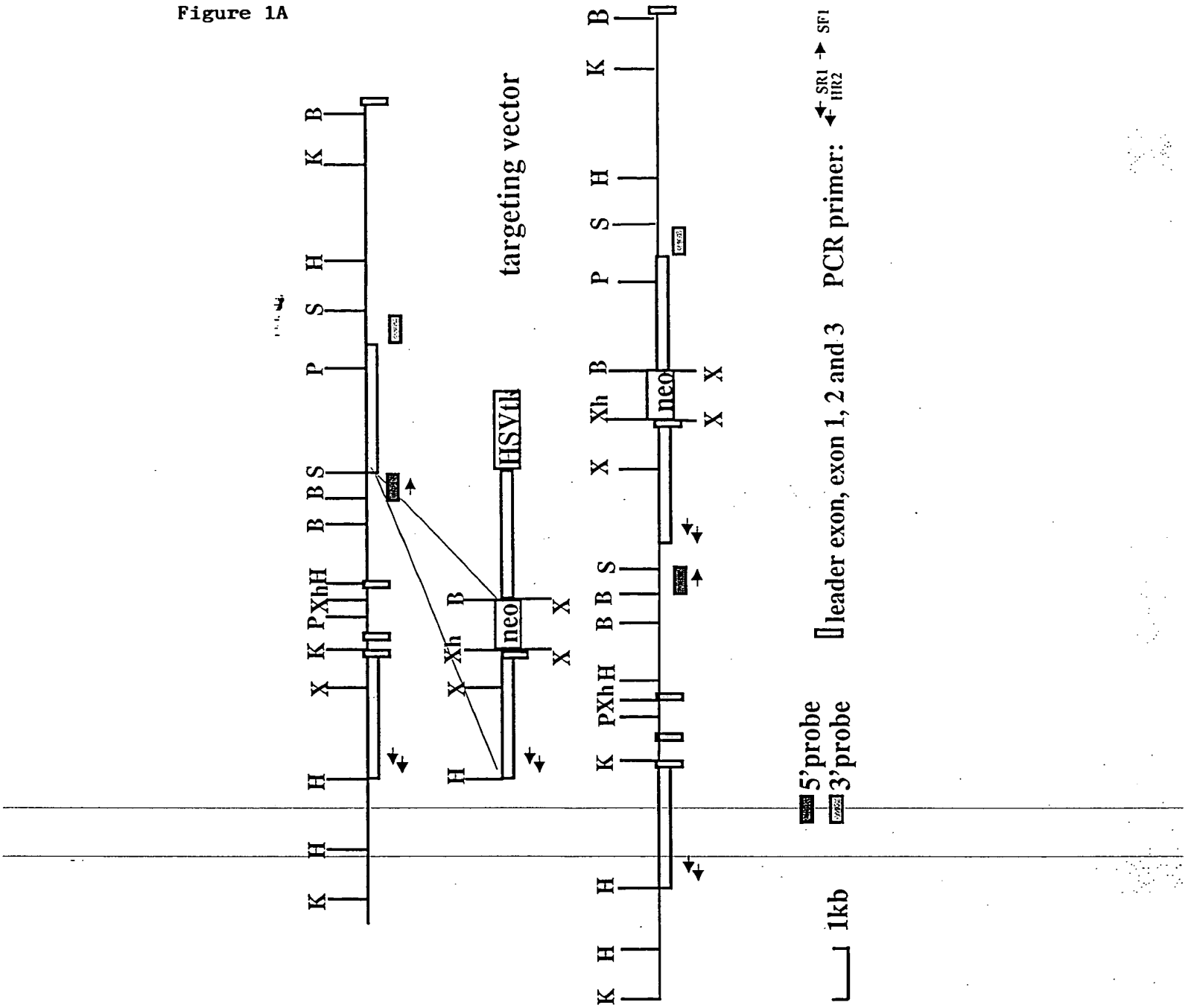
Figure 1A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neo
5'probe
3'probe
leader exon, exon 1, 2 and 3
PCR primer:
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HR2
SF1
1kb

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## *Vmat2* 5' probe

BamHI

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 51 GAGTGGTCAG ATCGTTCGTG GGATACTTTG CAAGTGGAAA ATGTGGGCCC
101 AGTGCTGAAA TGTTACAAAA CCTCAAAGAT ATCAACTGTA GCACAGAACT
151 AAATAAAGCC CAGAGAGGGA GGAACCCCGT GCTTCTTTCT GGTTCAGGGT
201 CTGTGCACTA AGGAGCCCCG GGGCGACCTC AGAGCAATCA GGGAGGGTAT
251 GGCAAGTCTT ACCACTGGGT GCAAATGTCT CCCCTCAGCA CCAGCTGTGC
301 TTGGGCATGT AGGCTGCTGC TGCTCTGCTC ACAGGGTGGC ACAGGGCACT
351 CCCTCCCTGT GGGTTGGGTG CTGCCACTGA GCCTGTGTTA ACAACCCTGG
401 CTTCAGACAG TCCTCCTGTC TTCTGAGCAG TACCGCATGG CTTCCATACA
451 AAGCATGGTA AGCAAATGAA CCTGGCATGG CTGATTGTGC ACCCACAGAG
501 AAGCCCCATT ATGGAGGTGA CACTGCTGTT CTCTGTTCTC CAGCACAAAA
          SacI
551 CCAAACAGAG CTC
```

## *Vmat2* 3' probe

```
          PVUII
  1 CAGCTGTTTG GGACAGGGAT CTCCCATCTA GACATCACTG GTGCGGGGAG
 51 GGAGGAGGGC AATGATCTCA CAGTGGGCTT CTCACACGGA AAGCAGAAGG
101 AACCAGGAGT TAGAGCATGC TCCTGTGAGC ATGGGGACTC ACACATAGGC
                                                    PVUII
151 AGGTCTGCTG TTCACACAGG TCCTTGTACA CATGTAGCCC AAACTCCAGC
201 TGACTCAAGG GCTGGACTTA AGGATTTCAT TTCATTTCCT AAATACTGAA
251 CAAACTTCCT GTTATAGCTG AGTGAATTTG AGATATTCCT GTTTCTTGAC
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Figure 1B

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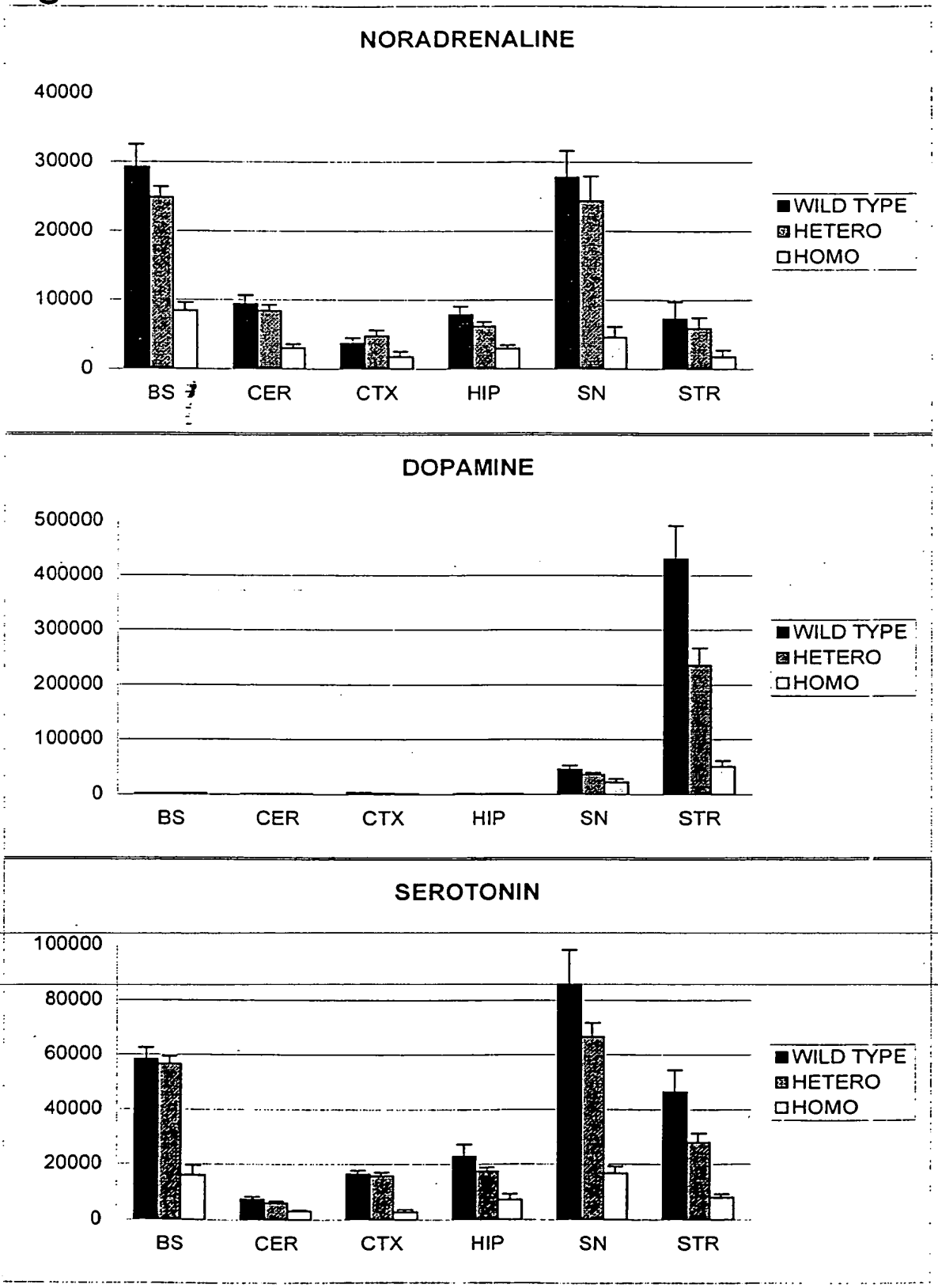
# Figure 2A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DOPAMINE
500000
400000
300000
200000
100000
0
BS
CER
CTX
HIP
SN
STR
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HETERO
HOMO
SEROTONIN
100000
80000
60000
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WILD TYPE
HETERO
HOMO

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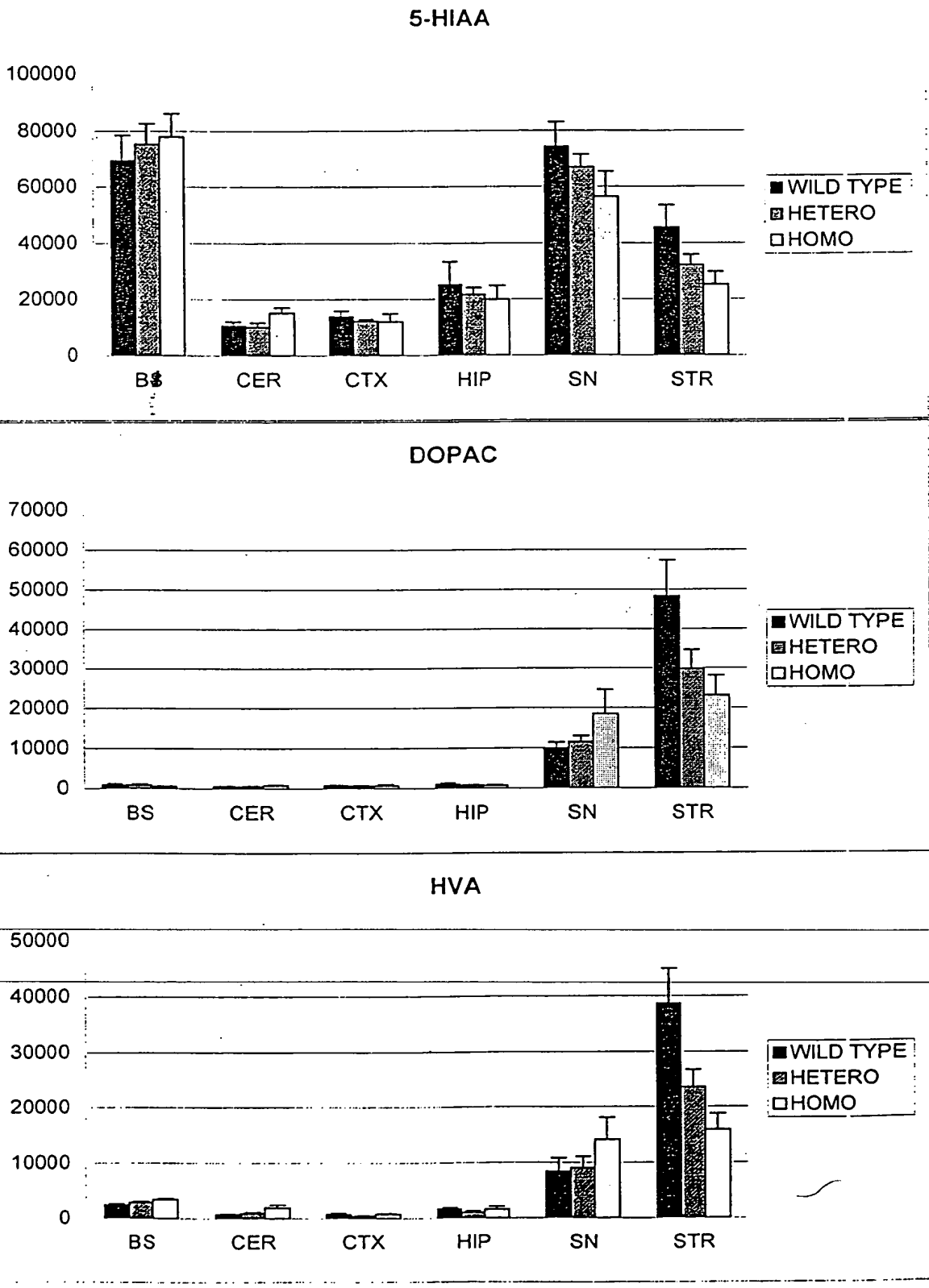
Figure 2B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DOPAC
70000
60000
50000
40000
30000
20000
10000
0
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CER
CTX
HIP
SN
STR
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HETERO
HOMO
HVA
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30000
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HETERO
HOMO

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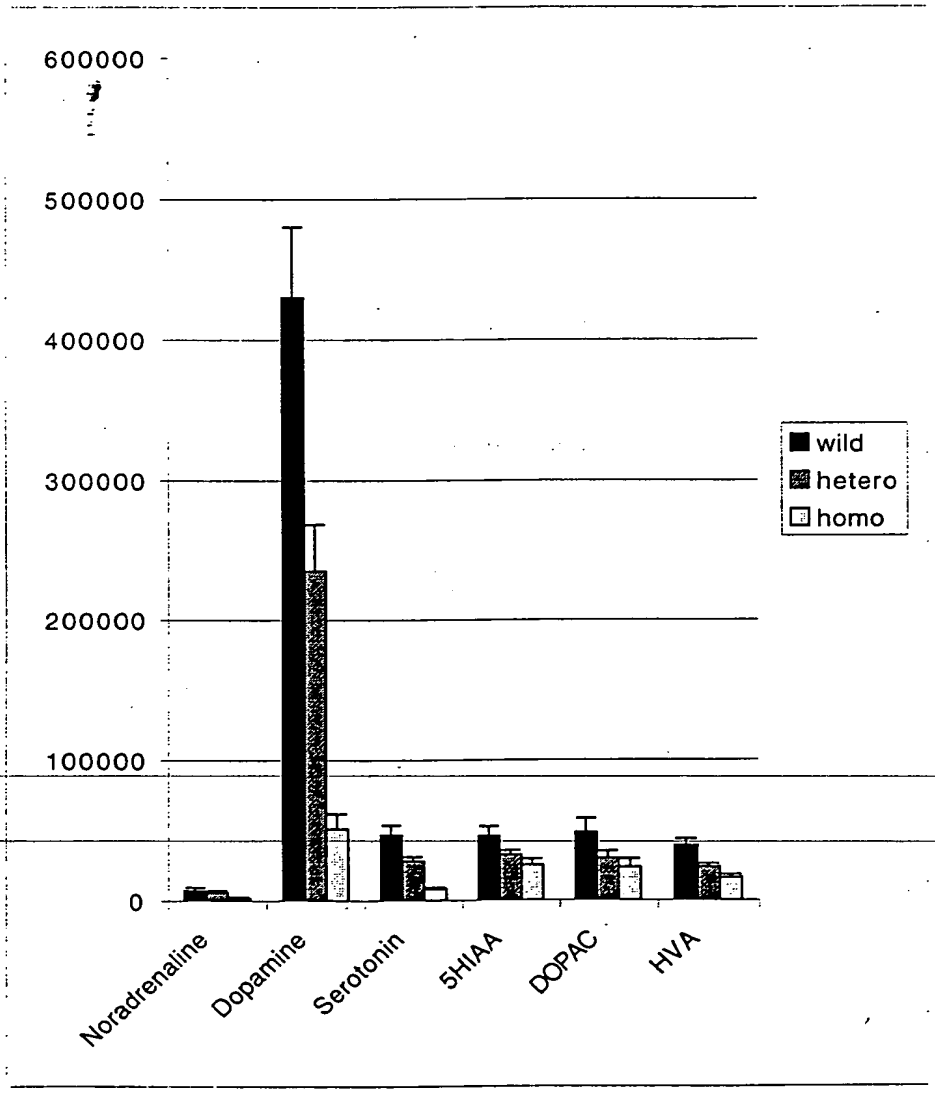
# Figure 3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hetero
homo
Noradrenaline
Dopamine
Serotonin
5HIAA
DOPAC
HVA

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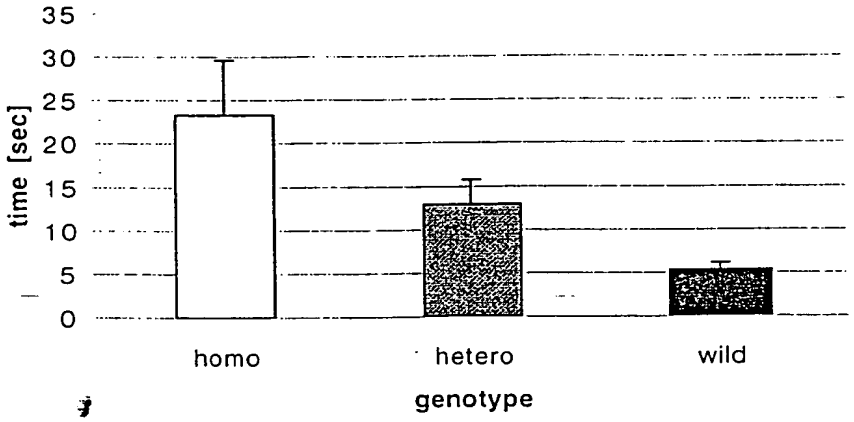
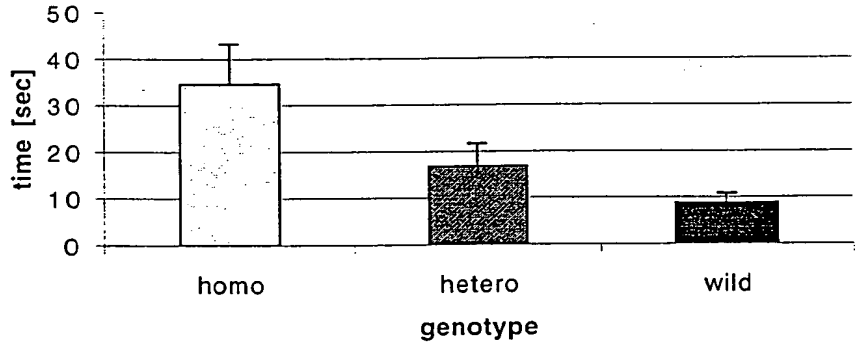
# Figure 4

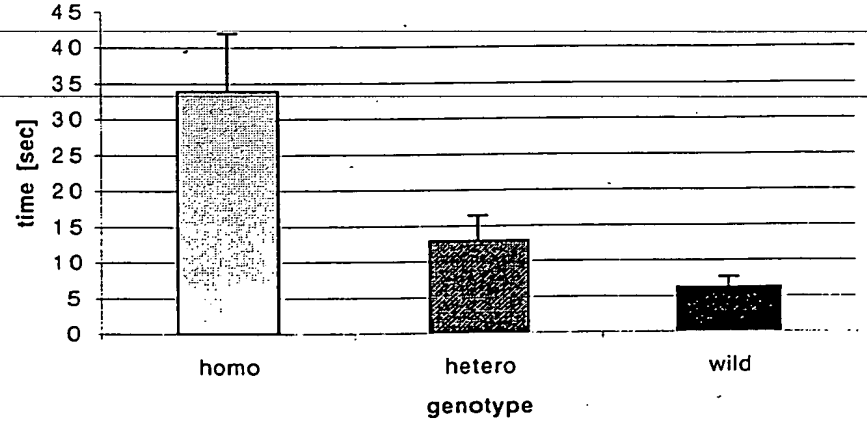
A


35
30
25
20
15
10
5
0
time [sec]
homo
hetero
wild
genotype

B


50
40
30
20
10
0
time [sec]
homo
hetero
wild
genotype

C


45
40
35
30
25
20
15
10
5
0
time [sec]
homo
hetero
wild
genotype

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# Figure 5

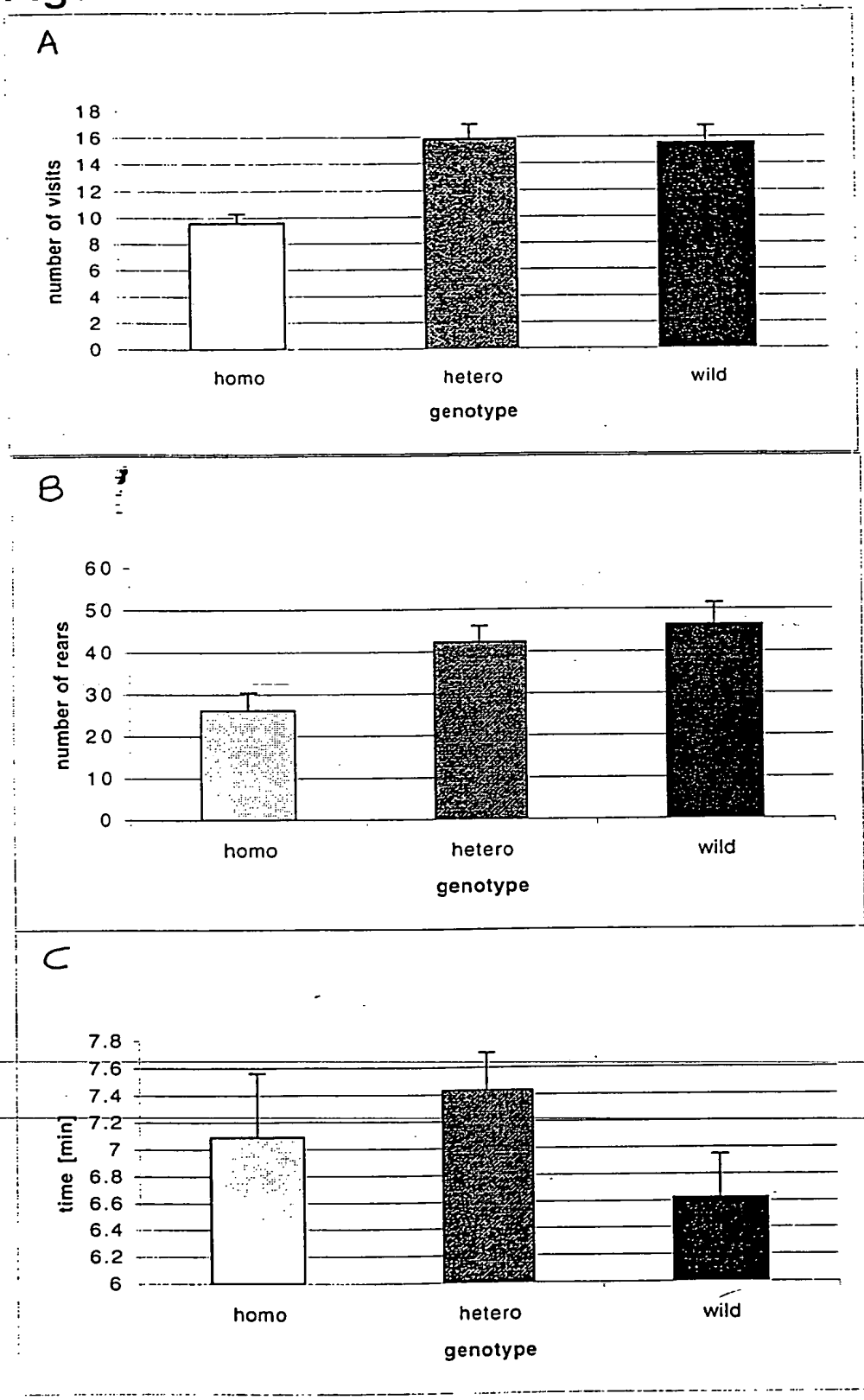
A
18
16
14
12
10
8
6
4
2
0
number of visits
homo
hetero
wild
genotype
B
60
50
40
30
20
10
0
number of rears
homo
hetero
wild
genotype
C
7.8
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
6
time [min]
homo
hetero
wild
genotype

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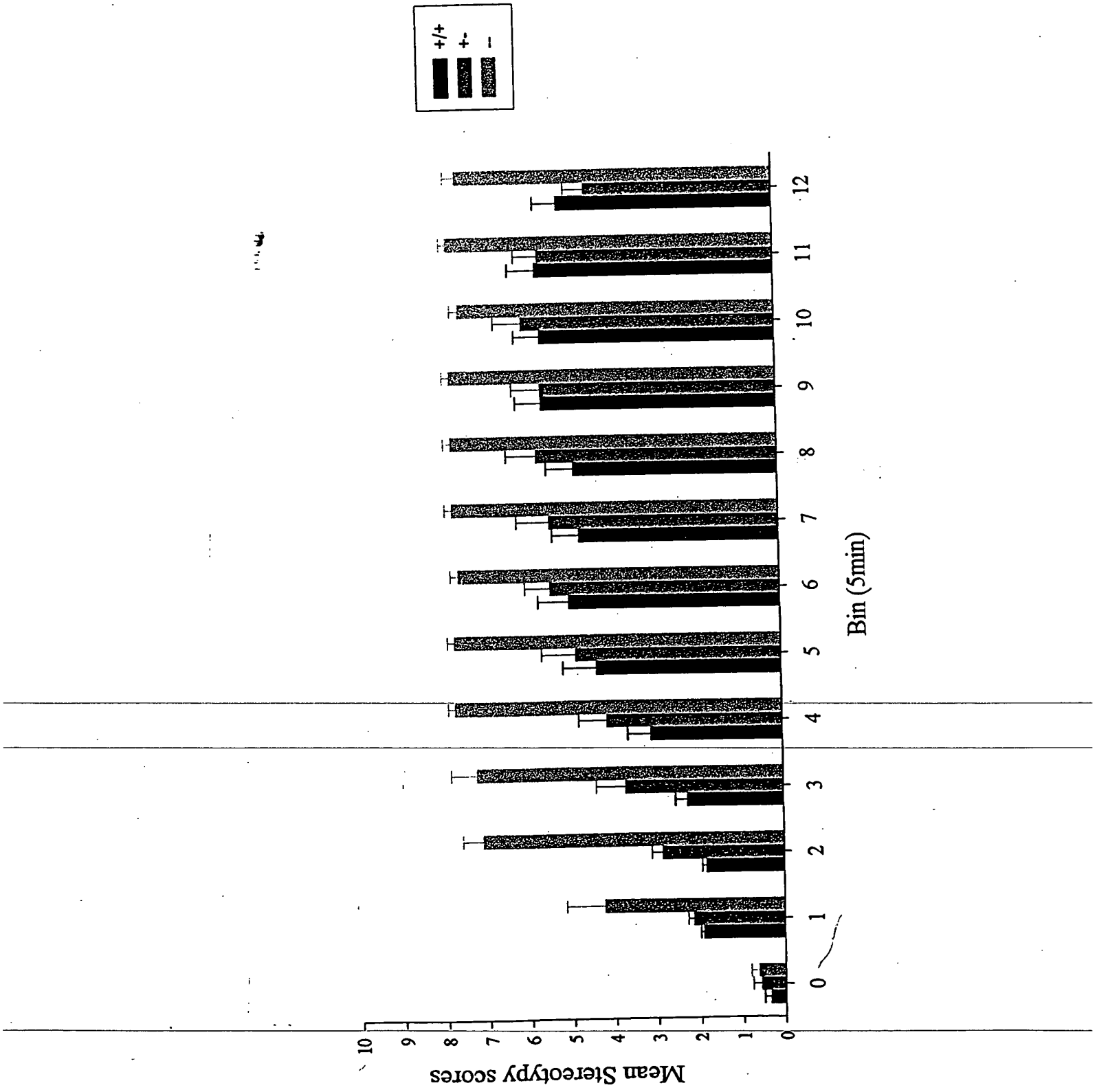
Figure 6


+/+
+-
--
Mean Stereotypy scores
0
1
2
3
4
5
6
7
8
9
10
Bin (5min)
0
1
2
3
4
5
6
7
8
9
10
11
12

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Mewburn Ellis

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